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Carbohydrate Polymers **24** (1994) 113–118

0144-8617/94/$07.00

Substitution patterns of water-unextractable arabinoxylans from barley and malt

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(Received 21 September 1993; accepted 22 October 1993)

Intact arabinoxylan polymer fractions from barley and malt differing in their ratio of arabinose to xylose (ara:xyl ratio) were degraded with a purified endo-xylanase (E.C. 3.2.1.8) from *Aspergillus awamori*. Enzymic degradability decreased with increasing ara:xyl ratio. No differences in degradability between corresponding fractions from barley and malt could be observed. The fragments liberated by enzymic action were characterized using HPAEC. Fragments consisting of less than seven pentose residues contained relatively low amounts of O-2- and O-2,3-substituted xylose, compared to the original substrate. Xylose residues substituted at O-2 or both O-2 and O-3 appeared to be concentrated in the larger fragments. From these results and from our knowledge of the mode of action of the xylan degrading enzyme it was concluded that substituted xylose residues were not randomly distributed in the polysaccharide, but were arranged according to a pattern in which isolated unsubstituted residues are separated by one or two substituted residues. This pattern was interrupted by sequences of contiguous unsubstituted xylose residues. From the available data it was concluded that those sequences could reach a length of at least four residues, but the presence of longer unsubstituted sequences could not be excluded. Simulations of arabinoxylan structure and its enzymic degradation supported this model.

INTRODUCTION

Arabinoxylans are one of the major non-starch polysaccharides (nsp) present in cereal endosperm and aleurone cell walls. They play an important role in the processing of cereals (e.g. baking and brewing) (Fincher & Stone, 1986). In brewing, an excess of arabinoxylans in barley malt or adjuncts (maize, wheat, rice) can cause processing problems like poor filtration rates or the formation of beer hazes and precipitates (Fincher & Stone, 1986; Letters, 1969).

In barley, the arabinoxylans comprise 20–25% of the endosperm cell wall polysaccharides (Fincher, 1975; Ballance & Manners, 1978) and 85% of the aleurone cell wall polysaccharides (McNeil *et al.*, 1975). They consist of β-(1 → 4)-xylans, substituted to varying extents with single arabinofuranose residues (Ara*f*) at O-2, at O-3 or at both O-2 and O-3 (McNeil *et al.*, 1975; Viëtor *et al.*, 1992).

As arabinoxylans are especially abundant in aleurone cell walls of barley, insufficient degradation of these polysaccharides during malting could impair release of cell wall and starch degrading enzymes into the endosperm. This would result in retarded degradation of the cell walls in germinating barley. Total degradation of the cell wall arabinoxylans requires the presence of several enzymes, e.g. endo- and exo-(1 → 4)-β-xylanase and arabinofuranosidase. These enzymes are induced during the malting of barley (Preece & McDougall, 1958; Taiz & Honigman, 1976). Degradation of arabinoxylans during malting was found to be only partial (Viëtor *et al.*, 1991).

The extent to which the arabinoxylans can be degraded, as well as other features such as solubility and interaction with other polymeric cell wall components, depend on the presence of substituents (mainly Ara*f*) and their distribution over the xylan backbone (McNeil *et al.*, 1975).

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Knowledge of this distribution would therefore help in understanding the properties and degradation of the arabinoxylans, e.g. during malting. Furthermore, such knowledge would also be helpful in optimization of the use of exogenous enzymes in processes in which arabinoxylans play a role, e.g. baking.

In previous studies, we determined the chemical composition of arabinoxylans extracted from barley and malt cell wall material (Viëtor *et al.*, 1992) and the structure of small oligomers formed upon digestion of these arabinoxylans with a purified endoxylanase (Viëtor *et al.*, 1994). The barley and malt arabinoxylans were shown to contain xylose residues substituted at O-2, O-3 or both O-2 and O-3 with Ara*f* (Viëtor *et al.*, 1992).

The aim of this study was to elucidate the distribution of arabinose residues over the xylan backbone. We studied the enzymic digestibility of various polymeric arabinoxylan fractions. From the results of these experiments, in combination with knowledge of the structures of small fragments formed by enzymic degradation of barley arabinoxylans (Viëtor *et al.*, 1994) and the results of computer simulations of arabinoxylan structure and degradation, a model for the distribution of arabinose substituents over the xylan chain was proposed.

EXPERIMENTAL

General

Arabinoxylans used were extracted from-unextractable cell wall material of barley and malt using a saturated solution of $Ba(OH)_2$ containing $NaBH_4$ (BE fractions) (Viëtor *et al.*, 1992). The barley and malt BE fractions were further fractionated by graded ethanol precipitation (fractions BE-*nn*, with *nn* indicating the ethanol concentration in % (v/v) at which the fraction was precipitated) (Viëtor *et al.*, 1992). Purified endoxylanase 1 (E.C. 3.2.1.8) from *Aspergillus awamori* (Kormelink *et al.*, 1993*a*) was used for enzymic degradation of arabinoxylan samples. Conditions of enzymic degradation and methods used for determination of sugar composition and methylation analysis were described before (Viëtor *et al.*, 1992, 1994).

HPLC analysis

A Dionex system was used for high performance anion exchange chromatography (HPAEC) as described elsewhere (Viëtor *et al.*, 1994). The column was eluted with a gradient of sodium acetate in 100 mM NaOH (starting at 0 mM sodium acetate, increase to 150 mM acetate in 10 min, further increase to 500 mM sodium acetate in 45 min) at a flow rate of 1 ml/min. For calculation of fragment distributions the responses per mole were assumed to be equal for the known components. Coding and structures of the known fragments are described elsewhere (Viëtor *et al.*, 1994).

For high performance size exclusion chromatography (HPSEC), TSK 40-XL, 30-XL and 20-XL columns (250 × 4 mm) were used in series and eluted with 0·4 M sodium acetate/acetic acid buffer pH 3 (0·8 ml/min). The eluent was monitored with refractive index detection.

Simulation of arabinoxylan chains

Simulations of arabinoxylan structure and degradation were performed using programs written in Turbo Pascal version 5.5 (Borland Corp.), running on IBM-PC compatible microcomputers. Chain lengths of 2000 xylose units were used, as this was deemed sufficient to obtain representative results in reasonable calculation times. Moreover, a chain length of 2000 xylose residues gave molar weights for the arabinoxylan chains within the range of experimental values for cereal arabinoxylans (Fincher & Stone, 1986).

For simulation of the arabinoxylan chains, all substituents of the xylose were assumed to be single α-arabinofuranose groups. Arabinoxylan chains were simulated by random assignment of one of the four possible substituted xylose residues (no substituents, one substituent at O-2 or O-3 or substituents at both O-2 and O-3) to each of the 2000 xylose positions in the chain. The probabilities for selecting each of these four possible units were set equal to the mole fractions of the corresponding xylopyranose residues in the actual arabinoxylan fraction. These mole fractions were determined by methylation analysis (Viëtor *et al.*, 1992) (total xylose = 1, Table 1).

For simulation of enzymic degradation of the generated chains a large number of attacks was assumed, each leading to zero or one hydrolysed xylose–xylose linkage. The specificity of the endoxylanase 1 was determined from the structures found in digests of barley and wheat arabinoxylans (Gruppen *et al.*, 1992; Viëtor *et al.*, 1994) and is described in detail elsewhere (Kormelink *et al.*, 1993*b*). Basically, we concluded that the endoxylanase 1 could hydrolyse a β-(1 → 4)-linkage only if the new reducing end consisted of an unsubstituted xylopyranose residue (Xyl*p*). The Xyl*p* attached to O-4 of this unsubstituted residue could be substituted at O-3, but not at O-2. The new non-reducing end Xyl*p* could be unsubstituted or substituted at O-2 and/or O-3. The enzyme was not able to remove a single unsubstituted Xyl*p* from the non-reducing end of an arabinoxylan chain or fragment. Oligomers smaller than xylotriose could not be degraded. Action of the enzyme near O-2-substituted xylopyranose residues was identical to action near O-2,3-disubstituted xylopyranose residues.

Calculations for the simulation studies (for both chain formation and enzymic degradation) were repe-

Table 1. Relative linkage composition for xylose residues (in mol% of total xylose) in known fragments in endoxylanase 1 digests and in parental arabinoxylan fractions

Fraction	Substrate*				HPAEC fragments†			
	4‡	2,4	3,4	2,3,4	4	2,4	3,4	2,3,4
Barley (experiment)								
BE	56	10	14	19	75	4	18	3
P2-void	45	13	15	27	—	—	—	—
BE-20	71	4	15	9	76	2	20	2
BE-30	65	6	14	15	74	2	21	3
BE-60	55	9	14	22	71	3	21	6
BE-70	33	15	18	33	73	2	19	6
Malt (experiment)								
BE	57	12	14	17	77	4	17	3
P2-void	35	22	13	30	—	—	—	—
BE-20	78	3	15	4	77	2	19	2
BE-30	73	—	20§	8	76	3	19	2
BE-60	61	10	14	16	74	4	18	4
BE-70	36	18	16	31	76	3	16	5
Barley (simulation)								
BE-20					71	2	22	3
BE-60					67	3	27	4
BE-70					62	4	27	5

*Calculated from Viëtor *et al.* (1991).
†Calculated from Table 1 using structures of the fragments as described by Viëtor *et al.* (1994).
‡Numbers indicate positions at which xylose residues are substituted.
§Sum of 2,4- and 3,4-xylose.

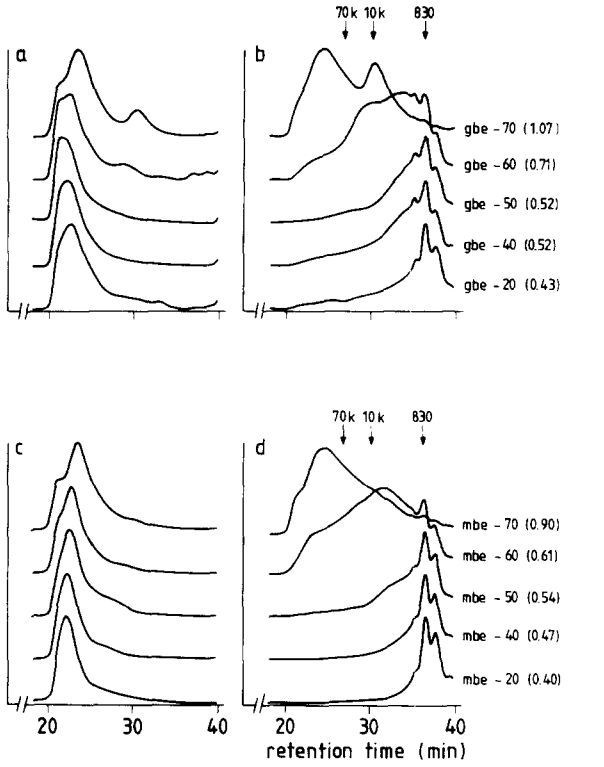

Fig. 1. HPSEC analysis of digests of arabinoxylans from barley and malt, degraded with endoxylanase 1 (a,b: barley, c,d: malt, a,c: blanks, b,d: digests; values between parentheses indicate the ara:xyl ratio of the fraction).

ated 10 times for each set of parameters, the results of each set of 10 runs were averaged.

RESULTS

Enzymic degradation of arabinoxylan samples

Figure 1 shows the results of HPSEC analysis of arabinoxylan fractions after digestion with endoxylanase 1. The extent of degradation of the arabinoxylans decreased with increasing ratio of arabinose to xylose (ara:xyl ratio). Arabinoxylans with ara:xyl ratios of 0·40 were almost totally degraded to small oligomers (retention time longer than 35 min) whereas samples with ara:xyl ratios above 0·9 showed little degradation. In all samples however, some small fragments were formed. Also the patterns in the low molecular weight region of the chromatogram (Fig. 1) were quite similar for all samples.

The results of HPAEC analysis of the oligomeric reaction products in the endoxylanase 1 digests (Table 2) were also very similar for all fractions. The relative proportions of the small fragments differ little between the various samples. The main differences between the barley and malt arabinoxylans were found in the fragments 5·1 and 6·1, which were present in larger relative amounts in the digests of the barley subfractions.

Comparison of the arabinoxylan fragments from barley shows that the relative amounts of the fragments 5·1, 6·1 and 6·3 increase with increasing ara:xyl ratio, and relative amounts of fragment 4·1 decrease. For the malt arabinoxylans, variations in relative amounts are mainly found for the fragments 3·1, 4·1, 5·1 and 6·3.

For fractions from both barley and malt, the amounts of xylose substituted at O-2 or at both O-2 and O-3 were quite small in the fragments analysed by HPAEC, much smaller than expected from methylation analysis of the undegraded parental polysaccharides (Table 1).

Methylation analysis of material collected in the void volume of a Bio-Gel P-2 column after separation of a BE-digest (fraction P2-void in Table 1) showed that 55% and 65% of the total xylose in these fractions were substituted for barley and malt, respectively. The material was enriched in O-2- and O-2,3-di-substituted xylose, compared to the parental polysaccharide. Incubation of the P2-Void fractions with fresh endoxylanase 1 did not yield any small fragments (data not shown).

Simulation of arabinoxylan structure and degradation

Simulations of arabinoxylan structures and digestion using a random distribution of substituted xylose residues, showed that the relative amounts of the various fragments as predicted from this model differ clearly

from the observed amounts (Tables 1 and 2). Also, variations in relative amounts of certain fragments with arabinoxylans show differences between the model arabinoxylans and the experimental data.

Table 2. Relative amounts by weight (%) of the main oligomeric degradation products in arabinoxylan digests of endoxylanase 1, measured by HPAEC (total of fragments 1–6·3 = 100)

Fraction	Fragment*										
	1	2	3·1	3·2	4·1	4·2	5·1	5·2	5·3 + 6·2	6·1	6·3
Barley (experiment)											
BE	7	16	11	7	14	9	11	6	5	11	2
BE-20†	10	17	9	8	21	3	8	6	5	12	1
BE-30	11	15	8	8	18	4	11	6	5	15	2
BE-40	10	16	8	7	19	4	11	6	5	16	1
BE-50	10	15	8	7	17	5	13	5	5	16	3
BE-60	10	13	7	7	14	6	20	8	4	19	7
BE-70	9	16	7	9	14	6	21	4	1	20	6
Malt (experiment)											
BE	7	21	11	5	19	7	10	5	6	9	1
BE-20	12	17	10	8	21	3	6	6	6	9	2
BE-30	11	16	10	8	20	6	8	6	6	10	1
BE-40	9	21	2	8	22	5	9	6	5	10	2
BE-50	12	18	6	8	19	6	10	6	5	9	3
BE-60	11	17	5	7	16	7	13	6	5	9	5
BE-70	11	19	5	7	15	6	15	4	5	8	5
Barley (simulation)‡											
BE-20	10	20	14	13	9	4	12	4	3	6	3
BE-60	12	16	8	11	5	7	20	4	4	6	8
BE-70	15	10	3	14	3	10	20	6	5	5	8

*Coding of fragments is described in Viëtor *et al.* (1994) and Fig. 2.

†Numbers indicate ethanol concentration at which the fraction precipitated.

‡Underlined values differ significantly ($P < 0.01$) from experimental values for the corresponding fraction (Student's t-test was used, with the standard deviation σ estimated to be 1 for all values given).

For instance in the simulation the relative amounts of the unsubstituted fragments 2 and 3·1 (xylobiose and xylotriose, respectively) decrease with increasing degree of substitution, whereas the experimental data show these to be more or less constant. Also, the random model predicts the presence of a large amount of fragments with DP > 6 in the digest, while the amounts actually found were relatively low (Viëtor *et al.*, 1994). These results show that a random distribution of substituted xylose residues does not give an adequate description of the arabinoxylans from barley and malt.

DISCUSSION

The fragments analysed by HPAEC (1–6·3) contained very little 2-O- and 2,3-di-O-substituted xylose. This implies that the larger fragments in the digests were substituted more than in the original substrate. This was indeed found for the barley and malt BE fractions (P2-Void). The degree of substitution of the P2-Void fractions from barley and malt was comparable to the degree of substitution of the corresponding BE-70 fractions.

A structural model for the arabinoxylans

As shown by the discrepancy in the relative abundances of oligomeric fragments estimated from experimental and simulation data, a random distribution of substituents over the xylan backbone does not give a good description for arabinoxylans from barley and malt WUS.

It was also demonstrated that the fractions BE-70 and P2-Void were hardly or not degradable by endoxylanase 1. This enzyme is able to hydrolyse the xylan backbone

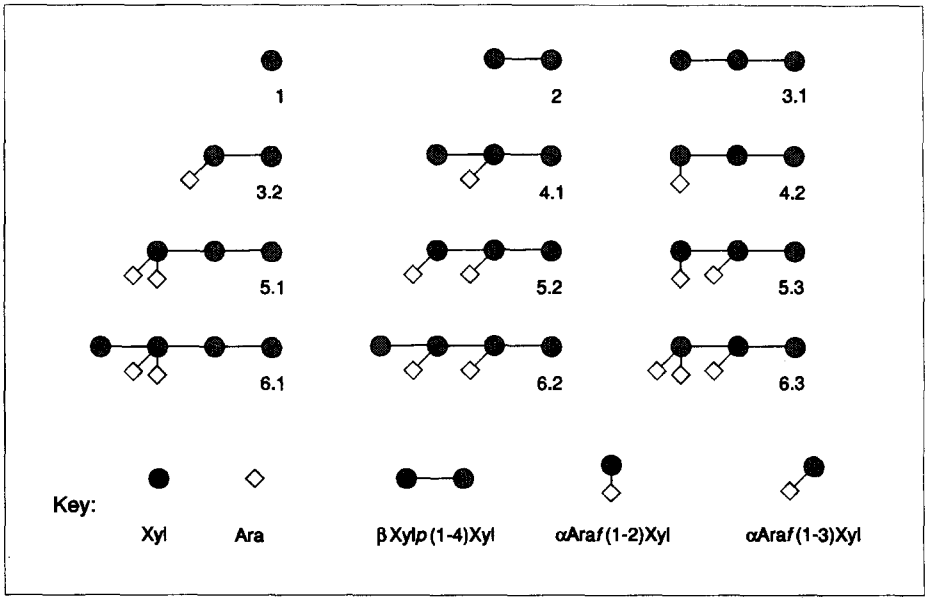

Fig. 2. Structures of arabinoxylan fragments isolated by HPAEC (Viëtor *et al.*, 1994).

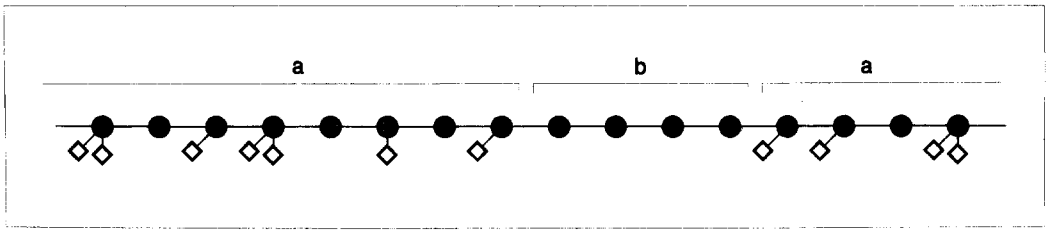

Fig. 3. Schematic view of a model for the distribution of substituents over an arabinoxylan chain as proposed in the text. Symbols are explained in Fig. 2.

at every position where two or more consecutive unsubstituted xyl*p*-residues are present (Experimental section and Kormelink *et al.*, 1993*b*). Therefore, sequences of two or more consecutive unsubstituted xyl*p* residues have to be absent or very rare in the BE-70 and P2-Void fractions. As the degree of substitution of these fractions was not very high (e.g. barley P2-Void: 55% of the xylose is substituted, Table 1), the distribution of the substituents had to be quite regular in these fractions.

At the same time, some sequences consisting of at least four consecutive unsubstituted xylose residues had to be present in the parent polysaccharides to account for the release of xylose, xylobiose and xylotriose from these substrates by endoxylanase 1.

This points to a structure containing two types of sequences (Fig. 3). The major type consists of isolated unsubstituted residues separated by one or two substituted residues (a in Fig. 3). This gives an average length of 1 xylose residue for the unsubstituted sequences and 1–2 for the substituted sequences. Blocks of this type were separated from one another by short sequences consisting of 2 or more unsubstituted xylose residues (b in Fig. 3). The presence of blocks of this latter type is required to explain the formation of xylose, xylobiose and xylotriose.

As the relative distributions of the fragments were very similar for all fractions analysed, the same model appears to be valid for the fractions with a lower degree of substitution. By varying the relative amount of sequences of several unsubstituted xylose residues (type b), variations in ara:xyl ratio can be explained.

The structure proposed above for the barley arabinoxylans closely resembles the structure proposed previously for wheat arabinoxylans (Goldschmid & Perlin, 1963; Gruppen *et al.*, 1993). In this model, the substituted xylose residues are present isolated or in pairs, separated by a single unsubstituted xylose residue. Blocks with sequences of this type are separated by sequences of two or more unsubstituted xylose residues. In their model, these latter sequences were the only site for hydrolysis by endoxylanases. We showed that another point of attack for the endoxylanase used here is a sequence formed by an unsubstituted Xyl*p* with a 3-O-substituted Xyl*p* attached at O-4. In this sequence, the unsubstituted residue will form the new reducing end after hydrolysis.

Comparing the arabinoxylans extracted from barley with those from malt, it is clear that the differences, if any are present, are very small. This was already observed during earlier analysis of these polysaccharides (Viëtor *et al.*, 1991, 1992, 1994).

ACKNOWLEDGEMENTS

This work was supported by The Netherlands programme for Innovation Oriented Carbohydrate Research (IOP-k) with financial aid from the Ministry of Economic Affairs and the Ministry of Agriculture, Nature Management and Fishery.

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